Assessment of gastrointestinal permeability by lactulose test in sheep after repeated indomethacin treatment

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ABSTRACT: The aim of the study was to assess the small intestine permeability by using lactulose as a sugar probe and blood metabolites in sheep after a challenge with repeated indomethacin injections. According to a change-over design, 7 adult sheep (4 males and 3 females) were subjected to 4 intramuscular injections (every 12 h) of saline [control (CRT); 7 animals] or indomethacin (INDO; 7 animals). Two hours after the last injection, 30 g of lactulose were administered orally to both CTR and INDO. Blood samples were collected daily for the analysis of the metabolic profile and 5 samples were collected at 2-h intervals following lactulose ingestion to monitor changes in blood levels of lactulose as an index of intestinal permeability. The INDO challenge induced clinical symptoms such as lack of appetite, dullness, weakness, depression, and diarrhea with traces of blood in the feces. In INDO group, haptoglobin and ceruloplasmin increased \( (P < 0.01) \) after INDO challenge whereas a decrease \( (P < 0.05) \) of negative acute phase reactants (e.g., cholesterol, albumin, and paraoxonase) was observed. Reactive oxygen metabolites increased \( (P < 0.01) \) from 60 to 204 h after the INDO challenge start, with a decrease of vitamin E concentration from 12 \( (P < 0.01) \) to 132 h \( (P < 0.05) \). Blood lactulose concentrations were increased \( (P < 0.05) \) in INDO animals and the highest mean values \( (17.67 \, \mu g/mL \text{ in INDO vs. } 0.17 \, \mu g/mL \text{ in CRT}; \ P < 0.01) \) were observed 6 h after oral dosage of lactulose. These changes indicate that the INDO challenge led to severe inflammatory responses with oxidative stress by enhancing small intestinal permeability in sheep that allowed lactulose to enter in blood. The results of this experiment demonstrate that lactulose can be used as a probe to assess gastrointestinal permeability in adult ruminants to test the consequences of stressing conditions on animal welfare. For this purpose, the most suitable time for blood sampling is between 2 and 8 h after the oral dosage of lactulose.

Key words: gastrointestinal permeability, indomethacin, lactulose test, sheep

INTRODUCTION

The maintenance of gastrointestinal integrity is essential for the proper absorption of nutrients and for the protection against the translocation of toxic substances such as lipopolysaccharides and microorganisms from the gastrointestinal tract to the blood stream. Increasing the intestinal permeability (IP) has implications for the incidence of various intestinal and systemic disorders. Intestinal permeability tends to be higher in humans with Crohn’s disease (Püspök et al., 1998), coeliac sprue (Vogelsang et al., 1998), or ulcerative colitis (Miki et al., 1998) and in ruminants due to acidic insults (Emmanuel et al., 2007; Klevenhusen et al., 2013). Intestinal permeability may also be increased with some chemical or physical stimuli, long-time fasting, prolonged and/or intense exercise (Pals et al., 1997; Lambert, 2009), hyperthermia (Lambert et al., 2002), and the administration of nonsteroid anti-inflammatory drugs (Suenaert et al., 2000; Gotteland et al., 2001). In humans and laboratory animals, IP is assessed noninvasively in vivo by measuring urinary recovery of orally administered lactulose (Bjarnason et al., 1995). Lactulose is neither digested nor metabolized by mammalian digestive enzymes and enters to circulation from the in-
testine through paracellular transport, which increases in response to mucosal damage (Hall, 1999).

In humans and laboratory animals indomethacin-induced enteropathy is a well-known and suitable experimental model to induce acute ileitis and to evaluate experimentally the effectiveness of the sugars permeability test (Murthy, 2006). To the best of our knowledge, there has not been any report to date on the use of lactulose to test IP in adult ruminants. This study aimed to verify if an indomethacin challenge can induce a damage of the gastrointestinal mucosa also in adult ruminants and if the associated increase in gastrointestinal permeability can be assessed by the lactulose test. Furthermore, we aimed to see the effects of a permeability increase on the systemic inflammatory response.

**MATERIALS AND METHODS**

This study complied with Italian laws on animal experimentation (DL number 116, 27/01/1992) and ethics.

**Animals and Feeding System**

Seven sheep (local population Bardigiana)—4 rams (69 ± 4 kg of initial BW) and 3 ewes (61 ± 3 kg BW)—were used in this experiment. Animals were housed in separate cages with wooden floor and water troughs in an environmentally controlled room (temperature 20°C and relative humidity 70%). Animals were fed twice daily (0700 and 1900 h) grass hay ad libitum (11.4% CP and 8.11 MJ/kg ME, DM basis), to ensure between 3 and 6% orts, and a vitamin–mineral supplement.

**Experimental Design**

The experiment was performed according to a changeover design with two 9-d lasting experimental periods (1 and 2) separated by a 4-wk interval. Animals underwent 2 treatments: intramuscular injections (4 injections at 12 h intervals) of indomethacin (INDO) or of normal saline [control (CRT)]. Indomethacin was dosed at 2.4 ± 0.07 mg per kg of BW for each injection.

Figure 1 depicts the time schedule of each experimental period. The first injection was done in the evening (time 0 = INJ_0) and basal (before morning meal) blood samples were collected at 12 h before (~12 h) and 12, 36, 60, 132, and 204 h after it. To assess IP, 2 h after the last injection of INDO or saline [lactulose dosage time (LDT)] 30 g of lactulose were orally dosed and blood samples were collected at 0, 2, 4, 6, 8, and 10 h thereafter.

**Feed Intake and Clinical Observations**

During the experimental periods feed intake, fecal characteristics, and general health status were continuously monitored. Orts were weighed every 12 h to monitor feed intake. Animals were weighed before feeding and 12 h before and 60 h after INJ_0.

**Blood Sampling and Analysis**

Blood was collected from the jugular vein into evacuated tubes containing lithium-heparin (Vacutainer; Becton Dickinson, Plymouth, UK) and immediately cooled in ice water. A small amount of blood was used for packed cell volume determination (Centrifugette 4203; ALC International srl, Cologno Monzese, Italy); the remainder was centrifuged at 3,500 × g for 15 min at 4°C and the plasma was frozen at −20°C until analyses.

Basal blood samples were analyzed to assess the following profiles: metabolic [glucose, total cholesterol, creatinine, urea, aspartate aminotransferase (AST/GOT), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), nonesterified fatty acids (NEFA), and β-hydroxybutyrate (BHBA)], mineral (Ca, P, Mg, Na, K, Cl, and Zn), and oxidative–inflammatory [total protein, albumin, globulin, total bilirubin, haptoglobin, ceruloplasmin, vitamins A, vitamin E, reactive oxygen metabolites (ROM), myeloperoxidase (MPO), thiol groups (SHp), total nitric oxide metabolites (NOX), and paraoxonase (PON)].

Blood metabolites were analyzed by an automated biochemistry analyzer (ILAB 600; Instrumentation Laboratory, Lexington, MA). Total protein, albumin, total cholesterol, total bilirubin, triglycerides, creatinine, urea, Ca, P, Mg, AST/GOT, GGT, and ALP were determined using kits purchased from Instrumentation Laboratory. Globulin was calculated as the difference between total protein and albumin. Ions (Na, K, and Cl) were measured by the potentiometric method (ion-selective electrode connected to ILAB 600). Zinc, NEFA, and BHBA were measured by the methods previously reported (Bionaz et al., 2007). Haptoglobin and ceruloplasmin were analyzed using the methods described in Skinner et al. (1991) and Sunderman and Nomoto (1970), respectively, adapted to the ILAB 600 conditions. The ROM and SHp were measured by commercial kits (Diacon International s.r.l., Grosseto, Italy). The MPO was measured spectrophotometrically.
according to the Bradley et al. (1982) procedure. The NOX were measured using the Griess test according to Gilliam et al. (1993) and Bouchard et al. (1999). The PON was measured by the method of Ferré et al. (2002) adapted to the ILAB 600 as previously described (Bionaz et al., 2007). Plasma vitamins A and E were extracted with hexane and analyzed by reverse-phase HPLC (Varian ProStar; Walnut Creek, CA) using an Allsphere ODS-2 3 µm, 150 by 4.6 mm column (Alltech, Deerfield, IL) and an UV detector set at 325 nm for vitamin A and at 290 nm for vitamin E; a solution of 80:20 methanol:tetrahydrofuran was used as mobile phase.

Lactulose Analysis

The blood samples collected to assess IP (0, 2, 4, 6, and 8 h from LDT) were analyzed for lactulose content using the Lactulose Assay Kit (Megazyme International Ltd., Wicklow, Ireland), adapted to the automatic biochemistry analyzer ILAB 600 (Instrumentation Laboratory).

Statistical Analysis

Data from each parameter was previously tested for normal distribution using the Shapiro test (SAS Inst. Inc., Cary, NC) and, when necessary, normalized by natural log transformation. Data were evaluated using the MIXED model analysis of variance procedure of SAS (release 8.0; SAS Inst. Inc., Cary, NC) using the REPEATED statement. The statistical model used as fixed factors treatment (INDO, and CTR) and time or hours (0, 2, 4 and 6) from Lactulose dosage time (LDT: $0_{\text{LDT}}$, $2_{\text{LDT}}$, $4_{\text{LDT}}$, $6_{\text{LDT}}$, and $8_{\text{LDT}}$), with interaction, and the individual sheep as random effect. Each parameter was subjected to 4 covariance structures—first order autoregressive, compound symmetry, spatial power, and toeplitz—and the best covariance structure was retained. The effect of the period (1 and 2) was initially included in the model and then removed being not significant for any parameter.

Statistical significance was established by using a conventional $P$ value of 0.05 or 0.01.

RESULTS

Health Status and Feed Intake of Animals

No symptoms of illness were observed in the CTR group. On the contrary, a strong reduction of appetite, dullness, weakness, and depression were observed in INDO sheep 12 to 24 h after the start of INDO injection. All animals recovered the normal behavior within 1 wk after the treatment end. The fecal consistency was also affected by INDO treatment. Indomethacin animals at 12 h had soft stools that became diarrheic at 24 h. In 4 of the 7 animals some blood in the feces could be observed from 36 to 48 h. The feces returned to be normal after 3 to 4 d from the INDO treatment end.

Average feed intake pattern is shown in Fig. 2. A rapid fall of feed intake was observed in INDO animals from 24 to 96 h from INJ_0. The lowest values were measured at 48 h (0.80 vs. 0.25 kg/12 h in CTR and INDO, respectively; $P < 0.01$).

Animals subjected to INDO injection also showed a significant loss of weight measured at 60 h after INJ_0 compared to CTR (–4.1 vs. –0.08 kg in INDO and CTR, respectively; $P < 0.01$).

Positive and Negative Acute Phase Proteins

Compared to CTR, INDO animals showed a quick rise of the positive acute phase proteins. Haptoglobin (Fig. 3A) reached its maximum value in INDO animals 36 h after the beginning of injections ($P < 0.01$) and remained higher in INDO until 132 h ($P < 0.05$). Ceruloplasmin (Fig. 3B) started to rise in INDO 60 h after the beginning of treatment ($P < 0.05$) and the values remained greater ($P < 0.01$) than in CTR up to 204 h after the first injection of INDO. Negative acute phase proteins showed a fall after challenge with INDO. Plasma albumin concentration (Fig. 3C) was lower in INDO animals at 60 ($P < 0.05$), 132, and 204 h ($P < 0.01$) after the beginning of challenge. A similar pattern of changes was also found in the plasma concentration of PON (Fig. 3D), significantly lower in INDO between 60 and 204 h after treatment ($P < 0.01$). Total cholesterol and vitamin A showed a similar trend (Fig. 3E and 3F); both decreased after treatment with INDO, reaching their minimum value at 36 h ($P < 0.05$) and then recovered to prechallenge values at 132 h. Total bilirubin showed an increment ($P < 0.05$) in INDO (Table 1), in particular from 12 to 60 h (data not shown).
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Indicators of Oxidative Stress

Among the parameters related to oxidative stress, the ROM rose in the INDO group from 36 h after the challenge. Differences became significant from 60 h ($P < 0.01$) and remained almost constant until 204 h after challenge (Fig. 4A). On the other hand, vitamin E concentration (Fig. 4B) started to decrease after 12 h from the challenge ($P < 0.01$) in INDO group and remained significantly lower until 132 h ($P < 0.05$). A significant increase of NOX (Fig. 4C) and decrease of SHp (Fig. 4D) were also observed in INDO. The highest value of NOX was observed at 36 h ($P < 0.01$) whereas SHp showed their significant reduction ($P < 0.01$) from 60 to 204 h of the experimental period.

Other Blood Parameters

Average plasma concentrations of some other parameters are presented in Table 1. Among them, a significant reduction in INDO was found for the concentration of Ca ($P < 0.05$) and ALP ($P < 0.05$) in comparison with the CRT group. Plasma concentration of urea increased significantly in INDO group and higher values were measured from 36 to 132 h ($P < 0.01$) compared to CTR (Fig. 5A). At the same time plasma creatinine concentra-

### Table 1. Average value of some blood parameters in animals subjected to 4 intramuscular injections of saline [control (CRT)] or indomethacin (INDO) solution. Significance of differences between groups at each time point is indicated by * ($P < 0.05$) or ** ($P < 0.01$).

<table>
<thead>
<tr>
<th>Item</th>
<th>CTR</th>
<th>INDO</th>
<th>SEM</th>
<th>Treatment</th>
<th>Hours</th>
<th>Treatment × hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV, 1 L/L</td>
<td>0.33</td>
<td>0.34</td>
<td>0.025</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.11</td>
<td>3.90</td>
<td>0.202</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca, mmol/L</td>
<td>2.69</td>
<td>2.52</td>
<td>0.096</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin, µmol/L</td>
<td>0.37</td>
<td>1.66</td>
<td>0.787</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP, 2 U/L</td>
<td>287.8</td>
<td>176.5</td>
<td>59.65</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1PCV = packed cell volume.
2ALP = alkaline phosphatase.

Figure 3. Effect of intramuscular injections of saline [control (CRT)] or indomethacin (INDO) solution on plasma haptoglobin (A), ceruloplasmin (B), albumin (C), paraoxonase (PON) (D), cholesterol (E), and vitamin A (F) (mean ± SE). The triangles (∆) indicate the injections. Significance of differences between groups at each time point is indicated by * ($P < 0.05$) and ** ($P < 0.01$). INJ_0 = time of first injection of indomethacin or saline.
tion (Fig. 5B) was significantly increased at 36 and 60 h after INDO challenge start \((P < 0.05)\).

**Intestinal Permeability Evaluation by Lactulose**

The lactulose was orally dosed 38 h after first injection; the pattern of lactulose concentration in blood plasma at different times after oral dosage is presented in Fig. 6. In the CRT group, lactulose concentration from 0 to 10 h after dosage ranged from not detectable to 0.41 µg/mL. In the INDO group it was not possible to detect any lactulose in blood from 0 to 2 LDT. At 4 LDT the blood concentration of lactulose started to rise and differed significantly from CRT \((P < 0.01)\), peaked (17.67 µg/mL) at 6 LDT, and then declined at 10 LDT (Fig. 6) although it was still higher \((P < 0.05)\) than in CRT.

**DISCUSSION**

Clinical manifestations of an acute phase response in ruminants have been often associated with an impairment of gastrointestinal permeability and consequent translocation of lipopolysaccharides (LPS) or bacteria from rumen or gut to bloodstream (Gozho et al., 2006; Khafipour et al., 2009; Plaizier et al., 2012). Acidotic insults of the gastrointestinal tract can in fact impair the gastrointestinal permeability and lead to LPS translocation into the bloodstream (Emmanuel et al., 2007; Klevenhusen et al., 2013). However, in field conditions there are no diagnostic tools to assess gastrointestinal permeability in ruminants. In humans and laboratory animals the IP is assessed noninvasively in vivo by measuring urinary recovery of orally administered lactulose (Bjarnason et al., 1995). This study was performed to test lactulose as probe to assess IP in adult ruminants with enteropathy experimentally induced by INDO.

In the present experiment the INDO was dosed at 2.4 mg/kg of BW for each of the 4 injections (9.6 mg/kg of BW for the entire treatment). The dose commonly used in laboratory animals is 7.5 mg/kg of BW on daily basis for 2 d (15 mg/kg for the entire treatment). At this dosage, in rodents INDO induced intestinal and colonic ulceration (Bernardes-Silva et al., 2004; Cury et al., 2008) whereas at a higher dosage and for more prolonged treatment the effect included necrosis and multiple perforations of small intestinal with ulcers and abscesses (Patel et al., 2002). Klein et al. (2007) successfully induced a gastrointestinal damage in calves by an oral administration of 0.5 to 3 mg/kg of BW of INDO. As adult ruminants can be supposed to be less sensitive than calves and the aim of this study was to induce macroscopic lesions in the gastrointestinal tract to assess lactulose IP test, we chose a higher dose of drug.

In animals treated with INDO, clinical signs of health problems were observed: fall of feed intake, diarrhea with visible blood in stools, weight loss, and lethargy. These observations are consistent with the litera-
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In fact, prolonged treatments with high doses of nonsteroidal anti-inflammatory drugs (NSAID) are associated with intestinal malabsorption, protein loss, and ileal dysfunction (Bjarnason et al., 1993). More recently it has been suggested that the use of NSAID increases the risk of acute diarrhea (Lanas and Scarpignato, 2006; Fortun and Hawkey, 2007). In extreme conditions the consequence can also be the death of the animals (Kimura et al., 1998; Patel et al., 2002; Taiwo and Conteh, 2008).

However, in our experiment all animals had completely overcome the problems (restored feed intake and normal feces features and vitality) within 1 to 2 wk after treatment. Furthermore, the blood parameters measured in CTR group at the beginning of period 2 (i.e., after restoration from the previous INDO challenge) were not different from those measured on the same animals in period 1 before (–12 h) the INDO challenge. These results support the complete overcome of the animals from INDO challenge and the absence of appreciable carryover effects.

The INDO challenge used in this study was effective to induce enteropathy. The treatment triggered strong inflammatory responses as shown by the increased concentrations of haptoglobin and ceruloplasmin, markers of acute phase reactions (Gruys et al., 2005). The signs of inflammatory response were already evident 36 h after INJ_0 as showed by the haptoglobin rise.

Based on blood markers a strong oxidative stress was evident, as already showed by Omatsu et al. (2009) in cultivated cells treated with INDO. We observed a fall of antioxidant metabolites such as vitamin E (reduced 12 h after treatment) and SHp (reduced 60 h after treatment). The rise of ROM (36 and 60 h after treatment, respectively) and NOX (36 h after treatment) occurred slightly later compared with vitamin E reduction. The connection between INDO and oxidative stress as a cause of enteropathy is also well known. Although the mechanisms of INDO action are not completely understood, oxygen and free radicals have been implicated in NSAID-induced injury, particularly that due to INDO (Vaananen et al., 1991; Basivireddy et al., 2003; Bernardes-Silva et al., 2004; Omatsu et al., 2009). The mechanism would involve the uncoupling of mitochondrial oxidative phosphorylation that, in turn, promotes oxygen radical damage (Bjarnason et al., 1993). This would agree with the blood changes recorded in the present experiment. Likely, the initial production of ROM and NOX, as a consequence of the INDO administration and gut mucosa damage, was temporarily counteracted by consumption of vitamin E and SHp, which declined, whereas ROM and NOX only later increased in blood.

The timeline of changes in blood metabolites indicates that changes of the indexes of inflammation oc-
occurred almost contemporary to the variation of the oxidative stress reactants. This support the hypothesis that the preliminary oxidative stress induced by the INDO challenge induced a minor variation of the blood parameters. The observed oxidative stress was mainly a consequence of the mucosa damage secondary to the INDO effect and also of the resulting inflammatory process caused by translocation of LPS and bacteria.

Lactulose is often used to assess IP both in human clinical studies of intestinal inflammation as well as in animal models. To date only 2 reports on the assessment of IP with the lactulose test has been published on preruminant calves (Branco Pardal et al., 1995; Klein et al., 2007), measuring lactulose in urine. From our results, the evaluation of small IP by lactulose test proved to be an appropriate, noninvasive, and sensitive method also in adult ruminants. When animals were treated with INDO, compared to CRT, they had an increased concentration of lactulose in blood. This supports the hypothesis that the INDO challenge impaired the gut epithelium integrity allowing the LPS translocation as above mentioned.

Moreover, this study confirmed our previous results (Ahmed et al., 2013) that lactulose can largely escape the rumen and reach the small intestine and consequently it can be used as marker for the assessment of gastrointestinal permeability also in adult ruminants. Gastrointestinal permeability is usually evaluated by measuring urinary excretion of orally administrated lactulose but few studies have tested lactulose in blood or serum (Oriishi et al., 1995; Cox et al., 1999) to IP evaluation. Our results suggest that also blood lactulose concentration can be used to assess IP by lactulose test and the use of blood instead of urine makes this method more suitable for field studies on livestock animals. The maximum concentration of lactulose in blood (Fig. 6) was observed 6 h after its dosage in 6 out of 7 animals, with a good agreement with blood inflammatory and oxidative metabolites and with changes of some fecal features. To assess small IP in sheep with this methodology the best time schedule should include blood sampling between 2 and 8 h after lactulose dosage.

Our results indicate that the challenge with INDO proved to effectively impair gastrointestinal permeability also in ruminants and it can be used to study the inflammatory and metabolic consequences of this impairment. The lactulose test can be used to assess the consequences of dietary or other stressing conditions on ruminant livestock welfare. The gastrointestinal permeability test can be performed in adult ruminants orally dosing lactulose and monitoring its levels in blood between 2 and 8 h after dosage.

**LITERATURE CITED**


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